AD_____

Award Number: W81XWH-08-1-0538

TITLE: Exploring Neurofibromin Function in a Yeast Model of NF1

PRINCIPAL INVESTIGATOR: Aaron Gitler, Ph.D.

CONTRACTING ORGANIZATION: The University of Pennsylvania
Philadelphia, PA 19104

REPORT DATE: November 2011

TYPE OF REPORT: Revised Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
November 2011	Revised Final	1 September 2008 - 31 August 2011

4. TITLE AND SUBTITLE
Exploring Neurofibromin Function in a Yeast Model of NF1

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-08-1-0538

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Aaron Gitler, Ph.D.

E-Mail: agitler@stanford.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Pennsylvania
Philadelphia, PA 19104

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

We hypothesized that the NF1 protein Neurofibromin has functions in addition to its Ras-GAP activity and proposed to develop a yeast NF1 model to define new functions as well as novel regulators of known functions. In this 3 year project, we categorized genetic hits that we recovered from yeast screens. We divided hits into Ras-dependent and Ras-independent categories. We have identified proteins that physically interact with IRA1 and IRA2 (the yeast homologs of human Neurofibromin). We have also focused on a novel genetic interaction between IRA1 and IRA2 and yeast genes involved in peroxisome formation and function in yeast. These studies will illuminate the cellular pathways that Ira1 and Ira2 regulate and how neurofibromin deficiency contributes to disease.

15. SUBJECT TERMS
NF1, yeast, IRA1, IRA2, ras, GTPase, TAP, purification, mass spectrometry, stationary phase, heat shock

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	14	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Introduction

Type 1 Neurofibromatosis (NF1) is a devastating human cancer syndrome, characterized by benign and malignant tumors of primarily neural crest origin. NF1 is caused by mutations in the NF1 gene, which encodes a large protein, called Neurofibromin. Neurofibromin is a large protein and has been shown to be able to function as a Ras-GTPase-activating protein (Ras GAP) to down-regulate ras signaling. Accordingly, NF1-deficient tumors show elevated ras signaling levels. Defining novel regulators of Neurofibromin's function will help to suggest therapeutic interventions. Because the Ras-GAP domain only comprises a small portion of the protein, we hypothesize that Neurofibromin has cellular functions in addition to its Ras-GAP activity. We have developed a yeast NF1 model to define NF1 disease mechanisms. Budding yeast, *Saccharomyces cerevisiae,* have two NF1-like genes, called IRA1 and IRA2.

In year one of the project, we generated *ira1Δ* and *ira2Δ* mutant cells and used these to perform genomewide genetic screens to identify, in an unbiased fashion, genes and pathways that interact functionally with Ira1 and Ira2 in yeast, and hopefully Neurofibromin in mammalian cells.

In year two of the project, to extend and complement the results from the genetic screens, we began isolating IRA1- and IRA2-interacting proteins. We have identified a list of 78 proteins that specifically interact with IRA1 and IRA2. In the final year of this project we focused on a potentially interesting new genetic interaction that we discovered in our yeast screen.

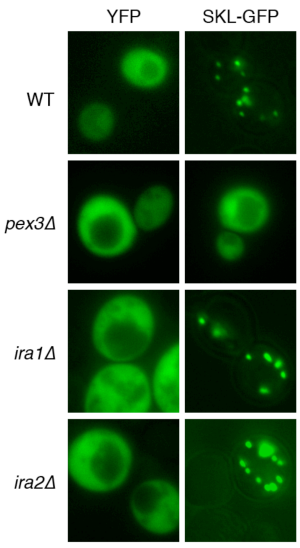

Figure 1. Testing the role of Ira1 and Ira2 on peroxisome biogenesis in yeast. We visualized peroxisomes using the SKL-GFP reporter. In WT yeast cells, peroxisomes formed and in *pex3Δ* cells they did not. Deletion of *IRA1* or *IRA2* did not affect peroxisome formation.

Body

We have defined a set of yeast genes that interact genetically with the *NF1* homologs, *IRA1* and *IRA2*, and have gone on to functionally categorize these genes as being either ras-dependent or ras-independent. Strikingly, we identified 17 Pex genes that interacted genetically with *IRA1*, *IRA2*, or both (Table 1). Pex genes are involved in the biogenesis of peroxisomes, evolutionarily conserved organelles present in almost all eukaryotic cells. They play a key role in the catabolism of fatty acids and the generation of cellular energy. Interestingly, *PEX3* is evolutionarily conserved and shares sequence similarity with human PEX3. The human peroxisome biogenesis disorders (PBDs) are a group of genetically heterogeneous diseases characterized by severe mental retardation, neuronal, hepatic and renal abnormalities, and death in early infancy. Mutations in *human* PEX3 have been associated with Zellweger Syndrome and Refsum Disease (Muntau et al., 2000a; Muntau et al., 2000b).

Since a connection between peroxisomes and neurofibromin has never been described, we sought to explore this connection further. We first tested if Ira1 and/or Ira2 are required for peroxisome biogenesis. We used a GFP-fusion protein that specifically labels peroxisomes (SKL-GFP) as a marker of peroxisomes. We introduced SKL-GFP into WT, *ira1*Δ and *ira2*Δ yeast cells (Figure 1). We also used *pex3*Δ cells as a positive control because it has been previously reported that peroxisomes do not form in this strain background (Hohfeld et al., 1991). Consistent with these reports, SKL-GFP was diffusely localized throughout the cytoplasm in *pex3*Δ cells (Figure 1). However, peroxisomes formed normally in WT, *ira1*Δ, and *ira2*Δ cells (Figure 1). Thus, Ira1 and Ira2 are not required for peroxisome biogenesis in yeast. Since *ira1*Δ *ira2*Δ double mutants are not viable, we were unable to examine peroxisome biogenesis when both neurofibromin homologs were mutated. Future studies will be aimed at defining whether neurofibromin plays a role in peroxisome function rather than biogenesis *per se*.

Table 1. Yeast Pex genes that interact genetically with *IRA1* or *IRA2* or both.

PEX Gene	Description	Synthetic lethal w/ Ira1	Synthetic lethal w/ Ira2
PEX 1	AAA-peroxin that heterodimerizes with AAA-peroxin Pex6p and participates in the recycling of peroxisomal signal receptor Pex5p from the peroxisomal membrane to the cystosol; induced by oleic acid and upregulated during anaerobiosis	yes	yes
PEX 11	Peroxisomal membrane protein required for peroxisome proliferation and medium-chain fatty acid oxidation, most abundant protein in the peroxisomal membrane, regulated by Adr1p and Pip2p-Oaf1p, promoter contains ORE and UAS1-like elements		
PEX 13	Integral peroxisomal membrane required for the translocation of peroxisomal matrix proteins, interacts with the PTS1 signal recognition factor Pex5p and the PTS2 signal recognition factor Pex7p, forms a complex with Pex14p and Pex17p		yes
PEX 15	Phosphorylated tail-anchored type II integral peroxisomal membrane protein required for peroxisome biogenesis, cells lacking Pex15p mislocalize peroxisomal matrix proteins to cytosol, overexpression results in impaired peroxisome assembly		yes
PEX 17	Peroxisomal membrane peroxin and subunit of the docking complex that facilitates the import of peroxisomal matrix proteins; required for peroxisome biogenesis		yes
PEX 18	Peroxin required for targeting of peroxisomal matrix proteins containing PTS2; interacts with Pex7p; partially redundant with Pex21p		
PEX 19	Chaperone and import receptor for newly-synthesized class I peroxisomal membrane proteins (PMPs), binds PMPs in the cytoplasm and delivers them to the peroxisome for subsequent insertion into the peroxisomal membrane		yes
PEX 2	RING-finger peroxin, peroxisomal membrane protein with a C-terminal zinc-		

	binding RING domain, forms translocation subcomplex with Pex10p and Pex12p which functions in peroxisomal matrix protein import		
PEX 27	Peripheral peroxisomal membrane protein involved in controlling peroxisome size and number, interacts with homologous protein Pex25p		
PEX 29	Peroxisomal integral membrane peroxin, involved in the regulation of peroxisomal size, number and distribution; genetic interactions suggest that Pex28p and Pex29p act at steps upstream of those mediated by Pex30p, Pex31p, and Pex32p		
PEX 3	Peroxisomal membrane protein (PMP) required required for the proper localization and stability of PMPs; interacts with Pex19p		yes
PEX 30	Peroxisomal integral membrane protein, involved in negative regulation of peroxisome number; partially functionally redundant with Pex31p; genetic interactions suggest action at a step downstream of steps mediated by Pex28p and Pex29p		
PEX 4	Peroxisomal ubiquitin conjugating enzyme required for peroxisomal matrix protein import and peroxisome biogenesis		yes
PEX 5	Peroxisomal membrane signal receptor for the C-terminal tripeptide signal sequence (PTS1) of peroxisomal matrix proteins, required for peroxisomal matrix protein import; also proposed to have PTS1-receptor independent functions		yes
PEX 6	AAA-peroxin that heterodimerizes with AAA-peroxin Pex1p and participates in the recycling of peroxisomal signal receptor Pex5p from the peroxisomal membrane to the cytosol		yes
PEX 7	Peroxisomal signal receptor for the N-terminal nonapeptide signal (PTS2) of peroxisomal matrix proteins; WD repeat protein; defects in human homolog cause lethal rhizomelic chondrodysplasia punctata (RCDP)		
PEX 8	Intraperoxisomal organizer of the peroxisomal import machinery, tightly associated with the lumenal face of the peroxisomal membrane, essential for peroxisome biogenesis, binds PTS1-signal receptor Pex5p		yes

As part of Task 2, we also pursued functional characterization of hits from the screens by Ras activity assays and also protein-protein interaction studies using TAP tag pull-downs. We reasoned that hits that also physically interact with IRA1 or IRA2 would be of particular value, since they could potentially serve as drug targets for manipulating the activity of NF1 or as buffers against the deleterious effects of NF1 mutation. Therefore, one major goal of the second year of our research project has been to identify IRA1 and IRA2 interacting proteins. The strategy we employed to identify IRA1 or IRA2 interacting proteins is coupling IRA1 or IRA2 purification with mass spectrometry identification of the co-purified proteins.

We employed C-terminal tandem affinity purification (TAP) tag to facilitate the purification of IRA1 and IRA2, since TAP tag purification involves two steps purification which are based on different principles, and therefore can dramatically diminish the non-specific background and give us highly purified protein complexes.

We optimized experimental conditions to purify TAP tagged IRA1 and IRA2 using untagged wild type strains as a negative control. This purification procedure was complicated by the fact that IRA1 and IRA2, like human NF1, are very large proteins. Both proteins contain more than 3000 amino acid residues and have a molecular weight more than 350 kDa. Further complications include the instability of the IRA proteins and their low expression level. We have been trying to avoid overexpressing IRA1 or IRA2 as that could lead to non-specific interactions irrelevant to the physical function of the IRA proteins and complicate the subsequent data analysis and interpretation. Using a TAP-tagged IRA2 strain, we succeeded in improving the purification efficiency and optimizing the growth conditions. We reasoned that we should be able to optimize the IRA2 expression by growing cell under conditions where IRA2 was known to be important for yeast cells to grow normally under those conditions. It has been known that IRA2 is important for yeast cells to enter stationary phase or for survival under heat shock condition. In our recent purification experiments, we used yeast cells grown to stationary phase or under heat shock conditions to purify TAP-tagged IRA2. We were able to increase the yield of the purification to a degree that we were able to see several specific protein bands on the SDS-PAGE gel when stained with Coomassie blue (Figure 2).

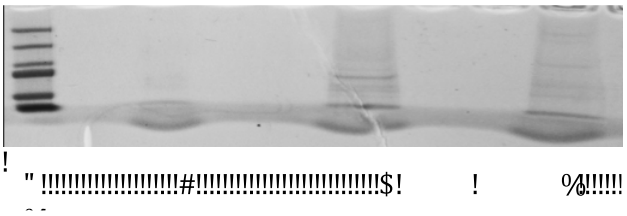

Figure 2. TAP-purification of IRA2-interacting proteins. Coomassie blue staining results of IRA2-TAP purification. Lane 1. Molecular weight Marker; Lane 2. WT control (stationary phase); Lane 3. IRA2-TAP (stationary phase); Lane 4. IRA2-TAP (heat shock treated)

We isolated these bands from the gel and subjected them to mass spectrometry in order to identify the associated proteins. This resulted in the identification of a total 78 proteins from stationary phase and heat shock treated IRA2 samples (identified at least five-fold more peptide fragments in one of the TAP tagged samples than in the untagged WT control sample, Table 2. Importantly, the protein GPB1, which has been previously reported to physically associate with IRA2 was also recovered from heat treated samples, indicating that the purification and mass spectrometry are indeed successful. This suggests that some of the additional proteins that we identified, if their interactions are validated, will likely also be relevant to NF1 function.

It is worthwhile to mention that the interacting protein profile of IRA2 between two growth conditions are very different with some interactions present more abundant in stationary phase cells and some interactions occur mainly in heat shocked cells. This difference might be reflecting different function/regulation of IRA2 under these two growth conditions. It also highlights the necessity to identify protein interactions under various different experimental conditions. It will be of great interest to determine those interacting proteins that

are in common to IRA1 and IRA2 as well as those that are specific to either IRA1 or IRA2.

Table 2. Hits form IRA2-TAP purification and mass spectrometry. Number of peptide fragments recovered is listed for the control strain (WT) and the IRA2 strains subjected to either stationary phase or heat shock.

WT (control)	Ira2 (stationary phase)	Ira2 (heat shock)	Interacting Protein
7	137	71	SSA2
1	97	175	IRA2
1	89	161	IRA2
1	49	142	URA2
1	33	15	HSC82
1	31	6	HSP104
1	30	1	DED1
1	25	28	RPL4B
1	23	11	PFK2
2	22	11	KAR2
1	21	19	SSA1
1	21	1	SSA4
1	19	13	CDC19
1	18	7	RPS7b
1	17	23	PFK1
1	16	6	GRS1
1	16	1	TIM44

1	15	1	YEF3
1	15	4	HOR2 OR RHR2
1	14	7	RPS3
1	13	9	CDC19
1	13	5	PMA2
1	13	3	ILV5
1	12	9	RPS0b
1	12	6	RPL13b
1	10	21	FBA1
1	10	9	PSA1
2	10	7	RPS1a
1	9	8	RPS14b
1	9	6	SSC1
1	9	6	YGL245W
1	9	1	FAS1
1	8	9	RPL2b
1	8	7	ADE5,7
1	8	6	RPS24a
1	8	3	RPS20
1	8	1	RPG1
1	8	1	YHR020W
1	7	8	RPS13
1	7	8	RPS18a
1	7	2	PAB1
1	7	2	RPL6b

1	7	1	RPL9a
2	6	11	MDJ1
1	6	2	CCT8
1	6	2	RPL16a
1	6	1	UBP3
1	6	1	YEF3
1	5	23	ATP3
1	5	15	RPL3
1	5	7	RPS6b
1	5	6	ILV2
1	5	6	HSP104
1	5	5	RPS1b
1	5	3	TEF4
1	5	1	PBP1
1	5	1	RPS22
1	4	16	ATP1
1	4	8	ACC1
1	4	7	RPL18b
1	4	6	ADH1
1	4	6	RPS15
1	4	6	TUB2
1	4	6	RPT5
1	3	106	GPB1
1	3	12	MIR1
1	3	7	HSP42
1	1	12	IDP2
1	1	11	YAT2
1	1	10	ACS1

1	1	8	IRA1
1	1	7	GDH2
1	1	6	ATP2
1	1	6	PET9
1	1	6	RPL10
1	1	5	HSP60
1	1	5	RPL23a
1	1	5	RPL7b

We next tested the effects of hits from our genetic screens on Ras activity by using ras activation pulldown assays. One of the well defined phenotype of ira1 deletion or ira2 deletion strain is the elevated RAS-GTP (active RAS form) content, which can be revealed by RAS pull-down assay using RAS-GTP specific binding protein Raf1-RBD conjugated to GST beads. However, it is very time-consuming and not cost-efficient to analyze the RAS-GTP content for so many hits by pull-down assay. It has been reported that domain-active RAS mutant has defect in glycogen storage which can be detected by iodine solution staining. To determine whether these hits from the screen actually involved in the RAS signaling pathway, we first used iodine staining to further screen hits that had glycogen storage defects (Figure 3). Among 118 hits from deletion library screen for ira1, 38 hits were stained lighter than WT by iodine, indicating a glycogen storage defect. For ira2, 36 out of 135 deletion library screen hits show lighter iodine staining than WT. From TS library screen hits, 23 hits of ira1 and 27 hits of ira2 show lighter

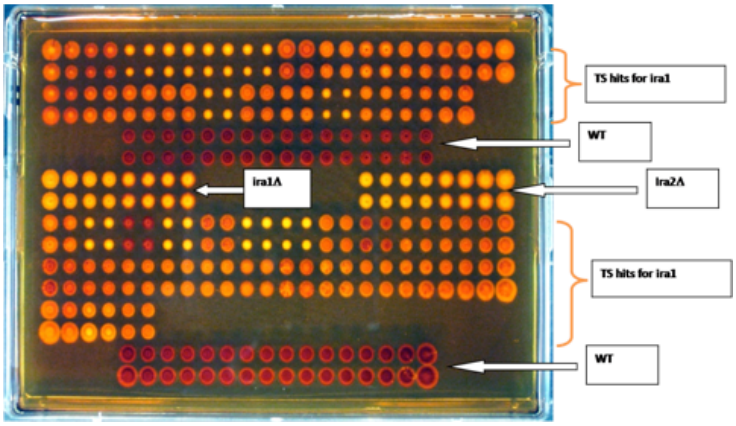

Figure 3. Iodine staining of ira1 and ira2 TS library screen hits

iodine staining than WT. These hits most likely involved in the RAS signaling pathway (Figure 3).

To further define the position of these iodine staining hits in the RAS signaling pathway, we performed RAS-pull-down assay to analyze the RAS-GTP content in these mutant strains. The original RAS-pull down assay kit was developed for mammal cells. We have modified the kit so that it can be apply to yeast RAS assay and the protein sample concentration can be normalized, therefore the ras-GTP content can be compared. As expected, RAS pull-down assay is very sensitive to GTP hydrolysis and not suitable to analyze large number of samples. We have to limit the number of sample for each assay to ensure the assay works. Right now, we have finished RAS-pull-down assay for 7 deletion hits and 12 TS mutant hits. Among these hits, we have found wsc3D, act1-136, cdc24-2, cdc24-5, and arp3-31 seem to have higher RAS-GTP content (Fig. 4).

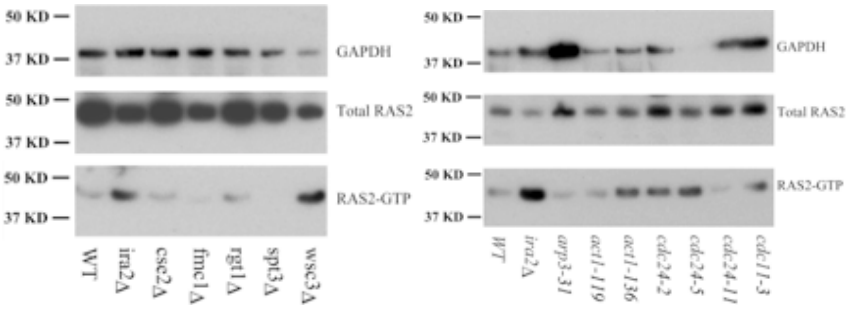

Figure 4 . Active ras pull-down assay on yeast genes that interact with ira1 or ira2. A GST-tagged Ras-GTP-binding domain of RAF was used to pull-down RAS-GTP. Immunoblotting with an antibody specific for yeast RAS2 was used to compare the relative levels of RAS-GTP to total RAS. Deletion *wsc3Δ* showed potent elevation in GTP-bound Ras.

Our preliminary data shows that arp3-31, act1-136, cdc24-2, cdc24-5 have slightly higher RAS2-GTP content. All these protein are well known to be involved in actin filament organization. These data suggest that there is a connection between RAS activation and actin filament organization. In the TS library screen, multiple act1 TS mutant (act1-108, actin1-119, act1-122, act1-25, act1-136) and cdc24 TS mutant (cdc24-1, cdc24-2, cdc24-5, cdc24-3, cdc24-11) all show strong alleviating genetic interaction with ira1 or ira2 deletion strains. How these two biological process are connected and how actin filament organization affect RAS-GTP content still need to be explored with more detailed assays.

Currently, our knowledge about RAS protein is that they can switch between GDP bound inactive form and GTP bound active form, and therefore serve as a molecular switch to transduction signaling. During RAS pull-down assay, we noticed that, in saturated cultures, Ira1 deletion strain and ira2 deletion strain show dramatically lower total RAS2 protein level compared with WT strain. Multiple experiments with ira2 deletion strain in different pull-down experiment set confirmed our observation (Figure 4). We have further confirmed the equal

loading of total protein in these samples by checking the GAPDH protein level. How do ira1 and ira2 regulate RAS2 protein level? What is the biology consequences and meaning for down-regulating RAS2 protein level? Interestingly, although wsc3 deletion, arp3-31, act1-136, cdc24-2, and cdc24-5 strains also affect RAS2-GTP content, they do not affect total RAS2 protein level.

Future directions: Finish RAS RAS Pull- down assay for the iodine staining positive hits. Check RAS protein level in all hits by western blot. Check RAS mRNA level by RT-PCR. Check RAS protein stability and mRNA stability in hits strains. Check IRA1 and IRA 2 interaction protein by TAP-tag purification and mass spectrometer.

Key Research Accomplishments

- Identified a novel cellular pathway (peroxisome biogenesis and function) that interacts genetically with neurofibromin homologs Ira1 and Ira2 in yeast.
- Determined that Ira1 and Ira2 are not required for peroxisome biogenesis.
- Identified genes that interact with ira1, ira2, or both.
- Identified proteins that physically associate with ira1, ira2, or both
- Determined the effect of ira1 and ira2 genetic interactors on cellular RAS-GTP levels

Reportable Outcomes

- Postdoctoral Fellow Xiaodong Fang, Ph.D., presented results from the genetic screen and proteomics study at the American Society for Cell Biology (ASCB) meeting:
  Authors: Xiadong Fang and Aaron D. Gitler
  Title of abstract: Yeast genetic screens to define mechanisms of neurofibromatosis type 1 (NF1)
  Location: Philadelphia, PA
  Date: December, 2010

Conclusion

In the 3 years of funding for our our project we have built off of our results from the genetic screens during year 1, developed a proteomics approach to identify yeast proteins that physically associate with NF1 homologs IRA1 and/or IRA2, and have focused on a novel cellular pathway (peroxisome biogenesis) that is possibly critical for neurofibromin function. These data will facilitate our continued mechanistic experiments aimed at identifying novel functions for neurofibromin as well as defining novel regulators of its known function as a regulator of the ras signaling pathway.

Future directions will be to test the role of peroxisomes and proteins involved in peroxisome formation and function on neurofibromin function in mammalian cells and in *Drosophila*.

References

Hohfeld, J., Veenhuis, M., and Kunau, W.H. (1991). PAS3, a Saccharomyces cerevisiae gene encoding a peroxisomal integral membrane protein essential for peroxisome biogenesis. J Cell Biol *114*, 1167-1178.

Muntau, A.C., Holzinger, A., Mayerhofer, P.U., Gartner, J., Roscher, A.A., and Kammerer, S. (2000a). The human PEX3 gene encoding a peroxisomal assembly protein: genomic organization, positional mapping, and mutation analysis in candidate phenotypes. Biochem Biophys Res Commun *268*, 704-710.

Muntau, A.C., Mayerhofer, P.U., Paton, B.C., Kammerer, S., and Roscher, A.A. (2000b). Defective peroxisome membrane synthesis due to mutations in human PEX3 causes Zellweger syndrome, complementation group G. Am J Hum Genet *67*, 967-975.

Appendices

None

Bibliography of publications and meeting abstracts

- Fang, X. and Gitler, A.D., Yeast genetic screens to define mechanisms of neurofibromatosis type 1 (NF1). Poster. The American Society for Cell Biology (ASCB) meeting in Philadelphia in December, 2010.

List of personnel receiving pay from the research effort

- Xiaodong Fang, Ph.D. (postdoctoral fellow)
- Aaron D. Gitler, Ph.D. (principal investigator)
- Andrew Elden, B.S. (research specialist)